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PRIMER NOTE

Isolation of polymorphic microsatellite markers from the malaria vector *Anopheles marajoara* (Diptera: Culicidae)

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Abstract

Microsatellite-containing regions were isolated and characterized in *Anopheles (Nyssorhynchus) marajoara*, a primary vector of malaria parasites in northeastern Amazonia, Brazil. An enrichment protocol yielded 500 positive clones. We designed primers to amplify 40 unique microsatellites, 11 of which amplified cleanly and were polymorphic. A survey of 323 individuals showed that these loci are highly variable with the number of alleles ranging from 11 to 52, and expected heterozygosity ranging from 0.64 to 0.95. These markers will be useful for studies of population structure and intraspecific variation in *A. marajoara*.

Keywords: Nyssorhynchus, neotropical, sex linked

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Anopheles (Nyssorhynchus) marajoara Galvão & Damasceno, ranges from southern São Paulo State, Brazil to Costa Rica, and was recently recognized as the primary vector of malaria parasites in northeastern Amazonia, Brazil (Conn *et al.* 2002). *An. marajoara* is a member of the albitarsis complex which includes four largely isomorphic species that can only reliably be separated using RAPD (random amplified polymorphic DNA) markers (Wilkerson *et al.* 1995a, 1995b) or by PCR (polymerase chain reaction) of diagnostic rDNA ITS2 sequence (Li *et al.* unpublished). Because of the significant vector status of *An. marajoara*, its wide geographical range, and questions regarding its taxonomic status, we developed microsatellite markers to study population and species level questions. This is only the second Neotropical malaria vector for which microsatellite markers have been developed, the other being *Anopheles (Nyssorhynchus) darlingi* Root (Conn *et al.* 2001).

Microsatellite loci were isolated using an enrichment protocol following Hamilton *et al.* (1999) with modifications as in Keyghobadi *et al.* (2004). Genomic DNA was extracted from three specimens of *An. marajoara* by phenol-

chloroform extraction as in Wilkerson *et al.* (1993) yielding approximately two µg of DNA which was digested with *Hae*III, *Mse*I, and *Nhe*I restriction enzymes (New England Biolabs – NEB). The resulting 200–1000 bp fragments were blunt-ended, dephosphorylated, and ligated to linkers as in Keyghobadi *et al.* (2004). This DNA was then 'enriched' for GT, GA, CAC, GTC, GGT, and GCT repeats using streptavidin-coated magnetic beads (Dynabeads), and cloned (Hamilton *et al.* 1999). Colonies were lifted with uncharged nylon membranes. DNA was fixed to the membranes, which were then hybridized to biotin-labelled (GT)₁₅ (GA)₁₅ (CAC)₁₀ (GTC)₁₀ (GGT)₁₀ and (GCT)₁₀ oligonucleotides at 65 °C, and positive colonies were detected using the Phototope-Star Chemiluminescent Detection Kit (NEB). Approximately 2000 colonies were screened, of which about one fourth were positive, and 150 positive colonies were picked, boiled in 100 µL TE for 10 min, and vortexed. After centrifugation for 5 min at 15 000 rpm, 1 µL of supernatant was PCR amplified with T3 (5'-AATTAACCCTCACTAAAGGG-3') and T7 (5'-GTAATACGACTCACTATAGGG-3') vector primers and directly sequenced using Big Dye 3.0 (Applied Biosystems – AB). Fragment analysis was conducted on a capillary automatic sequencer ABI3100 (AB). A blue colony (no insert) was used as a negative control in each PCR reaction.

For each microsatellite sequence with sufficient flanking sequence, flanking primers were designed with PRIMER 3

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Table 1 Characterization of 11 microsatellite loci in *Anopheles marajoara*

Locus	Primer Sequence (5'–3')	Repeat Motif	T_a (°C)†	No. of alleles‡	Allele size range (bp)	H_o	H_e	GenBank accession No.
M10-4	F: HEX-CACTTTCCGCTTCACTCCTC* R: AACAGTTTCGGCGGTCAAATC	(CA) ₂₁	56	52	211–265	0.79	0.95	AY741201
M1-3	F: TTTCATAATTATCCCGAAACGAA F1*: CATAATTATCCCGAAACGAACG R: FAM-GGCAACGATCTGCACTCTTAC	(GGT) ₇	50	15	196–221	0.58	0.83	AY741202
M11-4	F: GTCTTCACGACGACAACGAT R: NED-GTGTACCAGCGATGGTGTG	(TGA) ₅	50	16	195–226	0.51	0.68	AY741203
M5-7	F: HEX-CAATTGGTGAACGGTTTCCT R: CGACAGGGTGGTTAGTGGTT	(TGC) ₇	50	14	183–215	0.64	0.84	AY741204
M6-5	F: HEX-TGTPTGTCCACAGCAGAAG R: GCAGCAGATGCCATAAATCA	(TCG) ₇ + (TCG) ₃	50	13	132–172	0.69	0.81	AY741205
M2	F: ACCAGGGATCAGGGATCGT F1: ACCAGGGATCAGGGATCGT§ R: FAM-CACCGAGATCCTGCCGTA	(GGC) ₅ + (GAC) ₆	50	12	145–175	0.29	0.84	AY741206
M7-1	F: FAM-CTGGAAACCTGAAACCTGGA R: ACGGAACCTCTCTTTGTGA	(AC) ₁₃	50	17	141–169	0.59	0.83	AY741207
M10-1	F: NED-GCTGCCAATGAAAGATTGCT R: GTGTGCGTACACGAGCTACC	(AC) ₁₁	56	25	133–167	0.67	0.79	AY741208
M8-2-2	F: FAM-CAGGAGATGCCACAAATCAG R: AGAGACCAACTTTCGGATGC	(CGA) ₈	45	13	105–144	0.70	0.82	AY741209
M6-8	F: FAM-ATAGACCCCATTCGTCATCG R: TAGGTACGTCCATGGTAGCAGT	(TGC) ₁₀	50	11	100–133	0.56	0.64	AY741210
M9-2	F: CATACACTTCACACGTACGCACT R: FAM-AATCGGCACCTCCGCTTC	(GCA) ₇	50	11	87–119	0.42	0.66	AY741211

*F indicates forward primer and R indicates reverse primer; † T_a is the touchdown annealing temperature (touchdown protocol should start with a $T = T_a + 10^\circ\text{C}$); ‡Number of alleles, allele size range, and observed and expected heterozygosity (H_o and H_e , respectively), were estimated from a sample of 323 individuals from northern Brazil; §alternative primers for populations with null alleles.

(Rozen & Skaletsky 2000). DNA of individual mosquitoes for population studies was extracted as above (Wilkerson *et al.* 1993). The polymorphism of loci was tested by sizing PCR products from 12 individuals (selected from different localities) on 6.0% acrylamide gels (Protein 2 system, BIO-RAD) and visualized with ethidium bromide.

From the 40 loci for which primers were designed, we selected 11 polymorphic and easily scored loci for studying population differentiation (Table 1). One of the primers on each pair was labelled with FAM, HEX, or NED (AB). Touch-down PCR was conducted in a 20 µL reaction containing 1 × buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 2.5 mM MgCl₂, 200 µM dNTP, 5 pmol each primer, 0.5 U *Taq* polymerase (AB) and 0.5 µL DNA template (~5 ng). PCRs were performed on a GeneAmp PCR System 9700 (AB) with the following conditions: 2 min at 95 °C followed by 14 cycles using a touchdown protocol (95 °C for 30 s, 30 s at a temperature that decreased by 0.7 °C every second cycle to a 'touchdown' annealing temperature (T_a in Table 1), 72 °C for 30 s), followed by 25 cycles with a standard protocol (95 °C for 30 s, T_a for 30 s, 72 °C for 30 s), closing with 10 min at 72 °C to reduce stutter bands. Products from three to six microsatellite loci were mixed

and run on an ABI3100 (AB). Data were automatically collected and analysed by GENOTYPER software 2.5 (AB).

Linkage disequilibrium for all pairs of loci were tested with GENEPOP 3.3 (Raymond & Rousset 1995). After Bonferroni correction, one pair of loci was found to be significantly linked: M6-5 with M8-2-2. We compared observed and expected frequencies under Hardy–Weinberg equilibrium using ARLEQUIN (Schneider *et al.* 2000) with 100 000 steps in the Markov chain and 1000 dememorization steps. After Bonferroni correction, all loci conformed to Hardy–Weinberg frequencies except for M2. Upon further testing we concluded this locus is sex linked (occurs on the X-chromosome) as deviations from expected frequencies only occur in samples where both males and females were examined (as opposed to only females).

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